Neuron, Vol. 22, 53–62, January, 1999, Copyright ©1999 by Cell Press

BDNF Mediates the Effects of Testosterone on the Survival of New Neurons in an Adult Brain

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Summary

New neurons are incorporated into the high vocal center (HVC), a nucleus of the adult canary (*Serinus canaria*) brain that plays a critical role in the acquisition and production of learned song. Recruitment of new neurons in the HVC is seasonally regulated and depends upon testosterone levels. We show here that brain-derived neurotrophic factor (BDNF) is present in the HVC of adult males but is not detectable in that of females, though the HVC of both sexes has BDNF receptors (TrkB). Testosterone treatment increases the levels of BDNF protein in the female HVC, and BDNF infused into the HVC of adult females triples the number of new neurons. Infusion of a neutralizing antibody to BDNF blocks the testosterone-induced increase in new neurons. Our results demonstrate that BDNF is involved in the regulation of neuronal replacement in the adult canary brain and suggest that the effects of testosterone are mediated through BDNF.

Introduction

A vast surplus of new neurons is constantly produced in the ventricular zone of the adult canary brain, but only a fraction of these cells survive (Goldman and Nottebohm, 1983; Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1990b). Many of these new neurons are incorporated into the HVC, a large, anatomically discrete forebrain nucleus that is part of the songbird song system (Goldman and Nottebohm, 1983). The connectivity of this nucleus has been described, and HVC's involvement in the acquisition and production of learned song is well established (Nottebohm et al., 1976). Interestingly, new neurons recruited into the HVC replace older ones that die (Kirn and Nottebohm, 1993). We use the term "recruitment" to refer to the appearance of new, differentiated neurons in the HVC. This term does not discriminate between neurons drawn into the HVC and differentiating there or the survival of neurons after differentiation. Many of the neurons recruited into the adult HVC send long axons to another nucleus, the robustus archistriatalis (RA), which is part of the motor pathway for song production (Alvarez-Buylla et al., 1990a; Kirn et al., 1991). Some of the new HVC neurons, possibly the same ones that project to RA, also respond to sound (Paton and Nottebohm, 1984). These observations suggest that HVC neurons recruited in adulthood are integrated into existing auditory and motor circuits.

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What factors influence the survival of newly generated neurons in an adult brain? The recruitment of new HVC neurons in adult male and female canaries occurs throughout the year, but in males, this recruitment peaks in October and March, shortly after peaks in the number of pycnotic HVC cells (Kirn et al., 1994). Male canaries change their song every year, and this process of renewal also shows two peaks, in September-October and March-April (Nottebohm and Nottebohm, 1978; Nottebohm et al., 1986), raising the possibility that neuronal turnover is related to behavioral modification in this system (Nottebohm, 1989; Alvarez-Buylla and Kirn, 1997). The seasonality in the turnover of HVC neurons and song modification seen in males is thought to be influenced by blood testosterone levels (Nottebohm et al., 1987; Kirn et al., 1994). Drops in testosterone levels in males coincide with periods of unstable song and with cell death in the HVC (Nottebohm et al., 1987; Kirn et al., 1994), while rising testosterone levels accompany peaks in neuronal recruitment (Kirn et al., 1994). High testosterone levels in males accompany song crystallization (Marler et al., 1988), and exogenous testosterone induces male-like singing in female canaries (Nottebohm, 1980). Interestingly, the administration of testosterone to adult female canaries in the fall induces a 3-fold increase in the recruitment of new HVC neurons, and this occurs without an obvious increase in the rate of neurogenesis in the adjacent ventricular zone (Rasika et al., 1994).

Testosterone could affect the recruitment of HVC neurons in three ways: (1) it could act directly; (2) it could act indirectly, for example, by increasing the time the bird spends singing; and (3) it could act indirectly by upregulating the production of neurotrophic factors. These three routes need not be mutually exclusive.

BDNF, a member of the neurotrophin family, affects the survival of a variety of neuronal types during development and in adulthood (Barde et al., 1982, 1987; Lindsay and Peters, 1984; Johnson et al., 1986; Hofer and Barde, 1988; Alderson et al., 1990; Hyman et al., 1991; Knusel et al., 1992; Oppenheim et al., 1992; Segal et al., 1992; Sendtner et al., 1992; Yan et al., 1992; Friedman et al., 1993; Koliatsos et al., 1993; Mizuno et al., 1994; Acheson et al., 1995; Kirschenbaum and Goldman, 1995). It acts by binding to a specific receptor tyrosine kinase, TrkB (Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Barbacid, 1995; Chao and Hempstead, 1995), and both BDNF and TrkB are present in two areas known to incorporate new neurons in adult mammals, the hippocampus and olfactory bulb (Altman and Das, 1965; Kaplan and Hinds, 1977; Bayer et al., 1982; Ernfors et al., 1990; Hofer et al., 1990; Wetmore et al., 1990). In addition, BDNF has been shown to be regulated both by neuronal activity and by steroid hormones such as estradiol, a metabolite of testosterone (Zafra et al., 1990; Ernfors et al., 1991; Barbany and Persson, 1992; Castren et al., 1992, 1993; Falkenberg et al., 1992; Lindvall et al., 1992; Patterson et al., 1992; Dragunow et al., 1993; Herrera et al., 1993; Kokaia et al., 1993; Singh et al., 1995; Sohrabji et al., 1995); both androgen and estrogen receptors are present in HVC

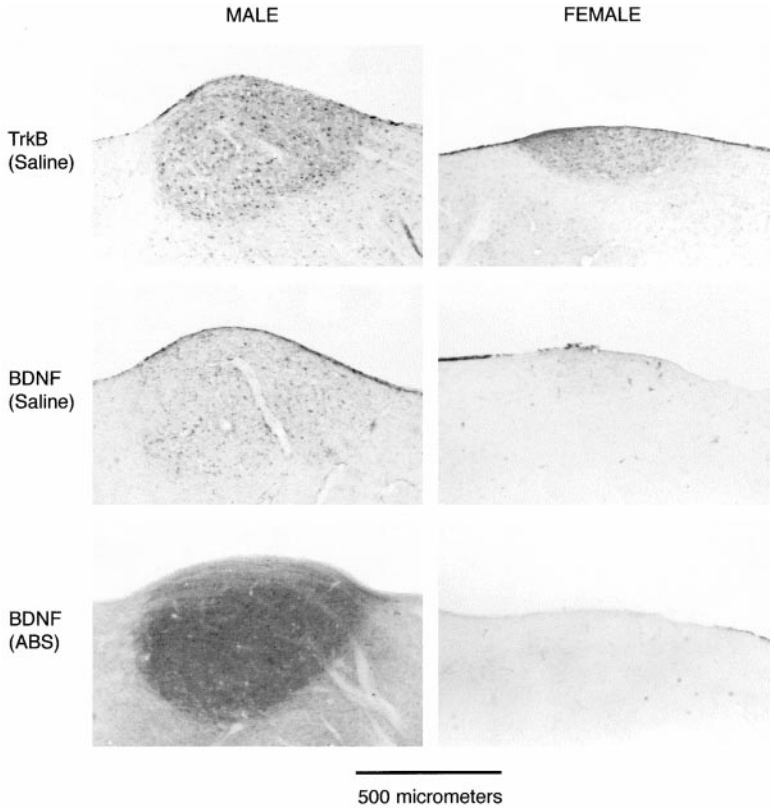

Figure 1. TrkB and BDNF Localization in Male and Female HVC

TrkB immunoreactive cells and neuropil are abundant in the HVC of both males and females, with only a few weakly stained immunopositive cells scattered outside the HVC. Cells that are weakly immunopositive to BDNF are present in the HVC of adult males perfused conventionally (i.e., with saline followed by paraformaldehyde). Immunostaining is dramatically increased after perfusion with acetate-buffered saline (ABS [pH 3.0]) and paraformaldehyde, although cellular morphology is lost. In contrast, very little BDNF immunopositivity can be seen in the HVC of adult females, regardless of perfusion method.

neurons (Arnold et al., 1976; Gahr et al., 1987; Sohrabji et al., 1989; Brenowitz and Arnold, 1990, 1992; Balthazart et al., 1992; Gahr et al., 1993; Johnson and Bottjer, 1993, 1995; Smith et al., 1996). Interestingly, BDNF mRNA expression in the HVC of adult male canaries appears to be increased by singing (Li et al., 1997), a behavior also dependent on testosterone (Marler et al., 1988). We therefore investigated the presence of BDNF and TrkB proteins in the HVC of adult male and female canaries and the role of BDNF in neuronal recruitment in this nucleus. Our results identify BDNF as a key player in the regulation of neuronal recruitment and survival in the adult brain.

Results

The Occurrence of TrkB and BDNF in the HVC of Adult Canaries

We carried out immunohistochemistry for BDNF and TrkB on parasagittal sections of the brains of adult male and female canaries killed in the spring and perfused with saline and paraformaldehyde (Figure 1). All animals were carefully monitored and prevented from singing for a period of 2 hr before perfusion, in order to minimize any effect that singing might have on BDNF protein levels, a precaution dictated by the observation that BDNF mRNA levels in the HVC are upregulated by singing and return to basal levels about 2 hr after the animals stop singing (Li et al., 1997).

Several regions were positive to BDNF and TrkB, and a complete inventory of these regions is shown elsewhere (Rasika, 1998). In the present report, we focus only on HVC and its immediately surrounding area. The HVC of both male and female canaries were found to contain cells and neuropil heavily immunopositive for the full-length or catalytic form of TrkB. Some areas outside the HVC, however, such as the shelf under the HVC, the caudal part of the hyperstriatum ventrale (cHV) and the caudomedial neostriatum (NCM), only contained scattered immunopositive cells, suggesting that these areas may not contain or respond to BDNF to the same degree as the HVC. Immunolocalization of BDNF in these sections revealed a marked sex difference in the HVC. Whereas many cells weakly immunopositive to BDNF were present in the HVC of adult male canaries, almost no BDNF immunostaining was observed in the HVC of adult females.

Given the abundance of the BDNF receptor, TrkB, and the fact that immunolabeling was carried out with a neutralizing antibody to BDNF (which competes with TrkB for BDNF), one potential reason for the low levels of immunostaining seen in the HVC could have been the masking of BDNF due to receptor binding. We therefore repeated the immunohistochemical protocol using sections from canary brains perfused with acetate-buffered saline (ABS) and paraformaldehyde. ABS has previously been shown to improve neurotrophin immunolabeling by dissociating the receptor from the neurotrophin (Zhou et al., 1994). BDNF immunostaining in the HVC of adult male canaries was found to be greatly enhanced by ABS treatment, suggesting that much of the BDNF in the HVC is normally bound to its receptor. However, ABS treatment destroyed cellular morphology in the HVC, and it was therefore not possible to determine if the

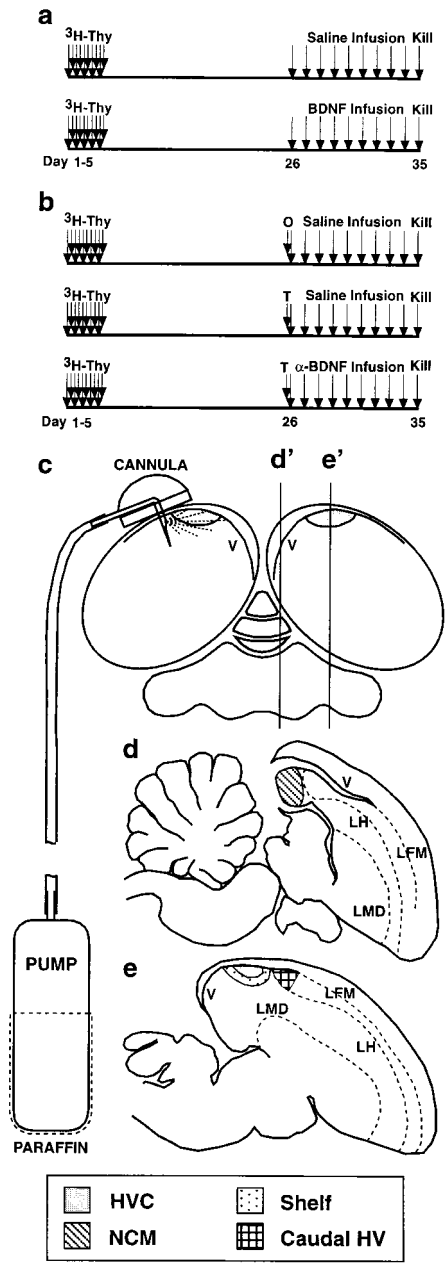

Figure 2. Diagram of Cannula Placement and Schematic of Protocols Used for the Infusion Experiments

(A) Protocol for the first infusion experiment. Two groups of female canaries were treated twice daily with injections of ^{3}H-thymidine for 5 days. Twenty-five days after the first injection, carrier (saline+BSA) or BDNF was infused for 10 days and the animals were killed.

(B) Protocol for the second infusion experiment. Three groups of female canaries were treated with injections of ^{3}H-thymidine twice daily for 5 days. Twenty-five days after the first injection, the first group received empty Silastic implants and infusion of carrier (saline+BSA), the second group received testosterone-filled Silastic implants and infusion of carrier, while the third group received testosterone-filled Silastic implants and infusion of α-BDNF into the HVC. Animals were killed 10 days after infusion began.

(C) Coronal section through the HVC, and the point of insertion of the cannula, with the bevel facing the HVC. The extent of the paraffin coating on the osmotic pump is also indicated.

(D and E) Representative parasagittal sections at the levels d′ and e′, respectively, shown in (C), to indicate positions of HVC and control areas mapped after BDNF infusion. Caudal HV—caudal part of hyperstriatum ventrale; LFM—lamina frontalis suprema; LH—lamina hyperstriatica; LMD—lamina medullaris dorsalis; NCM—caudomedial neostriatum; V—lateral ventricle.

receptor-bound BDNF was originally present within HVC cells, or in the extracellular space. BDNF immunolabeling in the adult female HVC was negligible even after ABS treatment suggesting that little, if any, BDNF is normally present in the female HVC, either in a free state or bound to its receptor.

BDNF Infusion Increases the Recruitment or Survival of New Neurons

We tested the effect of BDNF infusion on the HVC of adult female canaries in March and October. New neurons are born along the walls of the lateral ventricles in adult canaries (Goldman and Nottebohm, 1983; Alvarez-Buylla et al., 1990b) and, over a period of 20–40 days, migrate into and differentiate in the adult parenchyma (Alvarez-Buylla and Nottebohm, 1988). We injected adult female canaries systemically with ^{3}H-thymidine twice daily for 5 days to label a large contingent of new neurons, then waited 25 days from the first injection to allow the cells to migrate (Figure 2A). On day 25, we started infusion of either BDNF or saline immediately lateral to the HVC using an osmotic pump (Figure 2C). Birds were killed after 10 days of infusion. Cannulas did not result in lesioning of the HVC. The extent of the infusion could not be determined by immunohistochemistry for BDNF, possibly due to degradation or antigen masking in PEG embedded tissue. However, BDNF diffuses only a few millimeters in the mammalian brain parenchyma (Mufson et al., 1994; Sobreviela et al., 1996), which would have been enough in this case to cover the HVC and some neighboring areas, such as the shelf and cHV, but not to affect more distant regions. Neuronal identity was based on morphology in cresyl violet–stained sections. Cells containing large, regular, clear nuclei with one or two darkly staining nucleoli were considered to be neurons (Goldman and Nottebohm, 1983), and neurons with more than 20$\times$ background levels of silver grains were counted as ^{3}H-thymidine labeled (Figure 3).

BDNF infusion had several significant effects on the HVC (Figure 4). The HVC volume of the BDNF-treated females was larger than that of controls, by 58% in the spring and 68% in the fall (P-value for infusate = 0.0003; ANOVA); there were no significant volume differences with season (P-value for season = 0.5649; ANOVA). BDNF infusion increased the nuclear diameters of all HVC neurons from 6.77 to 7.41 μm in the spring and from 6.57 to 7.30 μm in the fall (P-value for infusate $<$ 0.0001; ANOVA). Nuclear diameters were also slightly but significantly smaller in the fall than in the spring (P-value for season = 0.0247; ANOVA). The packing density of all HVC neurons varied both with season and BDNF infusion (P-value for infusate = 0.0002; P-value for season $<$0.0001; ANOVA). In the spring, the packing density decreased from 210,803 cells/mm^3 in controls to 143,806 cells/mm^3 in BDNF-treated birds. In the fall, it decreased from 136,682 cells/mm^3 in controls to 108,456 cells/mm^3 in BDNF-treated birds. The total number of

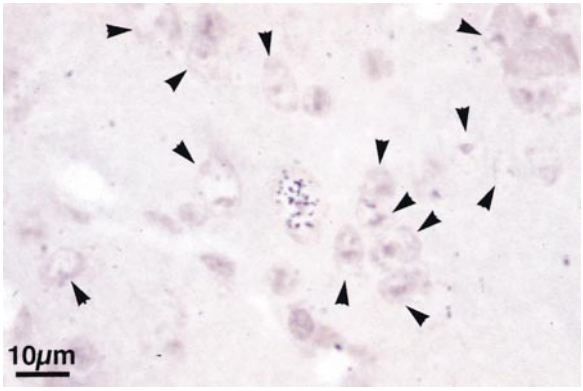

Figure 3. Photomicrograph of ^{3}H-Labeled and Unlabeled HVC Neurons in a Section Counterstained with Cresyl Violet

Note the ^{3}H-labeled neuron in the center with a large clear nucleus and the presence of numerous exposed silver grains that resulted from autoradiography of ^{3}H-thymidine. Unlabeled neurons in focus are indicated with black arrows.

HVC neurons increased from 15,690 in controls to 16,961 in BDNF-infused females in the spring (8%), and from 9,345 in controls to 12,517 in BDNF-infused females in the fall (33%), but these BDNF-induced differences were not significant (P-value for infusate = 0.1237; ANOVA). Interestingly, the total number of HVC neurons changed significantly with season (P-value for season = 0.0031; ANOVA).

The total number of ^{3}H-thymidine-labeled neurons increased 3-fold with BDNF infusion and was also greater in the spring than in the fall (P-value for infusate $<$0.0001; P-value for season = 0.0033; ANOVA) (Figure 4). The nuclear diameter of these neurons was not affected by season, but did increase with BDNF infusion, from 7.56 to 8.60 μm in the spring and from 7.36 to 8.73 μm in the fall (P-value for infusate = 0.0003; P-value for season = 0.8609; ANOVA). The packing density of ^{3}H-thymidine-labeled neurons increased in the spring from 3327 cells/mm^3 in controls to 5952 cells/mm^3 in BDNF-treated birds, and in the fall from 1998 cells/mm^3 in controls to 3857 cells/mm^3 in BDNF-treated birds (P-value for infusate $<$0.0001; P-value for season = 0.0004; ANOVA). There was no obvious gradient of ^{3}H-thymidine-labeled neurons in the HVC with respect to the infusion site, suggesting that the infused BDNF reached all parts of the HVC. Even though the absolute number of new HVC neurons was higher in the spring than in the fall, the percentage of new neurons in each of the two treatment groups (control and BDNF-treated) was similar in both seasons, respectively: 1.59% and 1.48% in the control group and 4.16% and 3.55% in the BDNF-treated group. Thus, the effect of BDNF was not season dependent, even though the physiological condition of the spring and fall females (onset of breeding season and end of molt, respectively) differed markedly.

The effect of BDNF on the packing density of new neurons was specific to the HVC, which contains TrkB, and did not occur, during either of the two times of year when this was tested, in neighboring regions—the shelf under HVC, cHV, and NCM (Figures 2D and 2E)—that lacked TrkB (Figure 5; P-value for infusate $>$0.1; ANOVA).

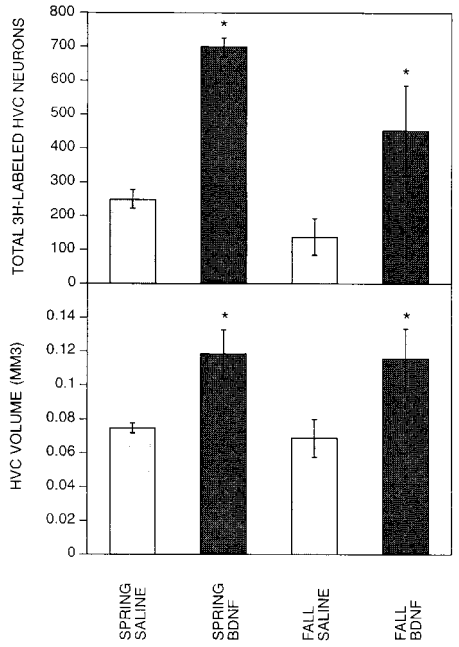

Figure 4. Effect of BDNF on the Total Number of New (^{3}H-Labeled) Neurons in the HVC and HVC Volume

An asterisk (*) indicates a significant effect of BDNF infusion (ANOVA). The total number of new or ^{3}H-labeled neurons triples with BDNF treatment, while the volume of the HVC increases about 60%, in both seasons.

The total number of new neurons in these regions was not estimated, due to the lack of morphological boundaries. It is interesting to note that the packing density of new neurons was in all cases considerably higher in the HVC than in the control regions studied. The nuclear diameter of new neurons in these regions was also not affected by BDNF (P-value for infusate $>$0.4; ANOVA).

Testosterone Increases BDNF Levels in the Female HVC

The sexual dimorphism in BDNF staining in the HVC suggests that BDNF levels may be affected by male-specific factors such as testosterone. This possibility, plus the observation that both testosterone (Rasika et al., 1994) and BDNF increase neuronal recruitment in the female HVC, led us to test whether testosterone might increase BDNF levels in the HVC. To this end, we treated adult female canaries with empty or testosterone-filled implants for 3 weeks in the fall (October), perfused these animals with ABS and saline 2 hr after they were prevented from singing, and carried out immunostaining for BDNF. As in the spring, the control females had a small, relatively flat HVC with little or no BDNF. In contrast, the testosterone-treated females had a larger HVC (see also Nottebohm, 1980; Rasika et al., 1994) and visible enhancement in BDNF levels (Figure 6). It appears, therefore, that testosterone can increase BDNF protein levels in the adult female HVC and be in part, at least, responsible for the sexual dimorphism in BDNF levels seen in untreated adult canaries.

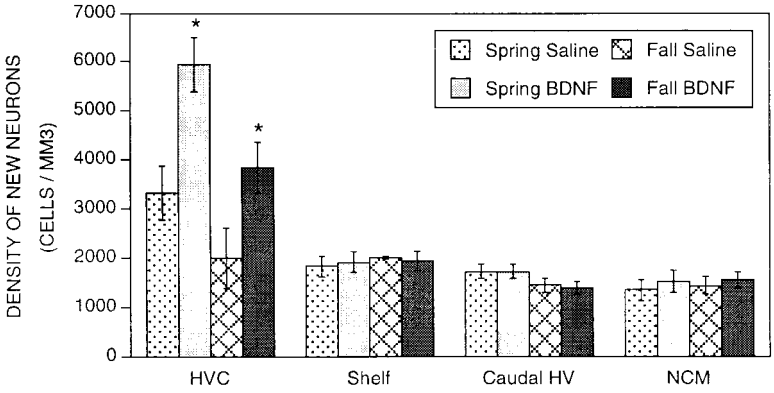

Figure 5. Effect of BDNF Infusion on the Density of New (^{3}H-Labeled) Neurons in the HVC and Three Control Regions

An asterisk (*) indicates a significant effect of BDNF infusion. BDNF infusion significantly increases the density of new neurons in the HVC in both seasons but does not change the density of new neurons in the control regions: the shelf under the HVC, caudal HV (hyperstriatum ventrale), and NCM (caudomedial neostriatum), in either spring or fall.

Neutralizing Antibodies to BDNF Block Testosterone-Induced Neuronal Recruitment in HVC

Adult female canaries normally have low serum testosterone levels (Weichel et al., 1986) and no appreciable levels of BDNF in the HVC. Testosterone treatment increases both the amount of BDNF in the HVC, as seen above, and the recruitment/survival of new neurons (Rasika et al., 1994). Therefore, if testosterone were to act through BDNF to affect neuronal survival, it should be possible to block the effects of testosterone by blocking BDNF. Accordingly, we injected adult female canaries in the fall with ^{3}H-thymidine twice daily for 5 days, in order to label a large contingent of new neurons (Figure 2B). After allowing 25 days for newly labeled neurons to migrate into the HVC, we started three treatment protocols. Birds in one group received empty Silastic implants and infusion of carrier solution through a cannula implanted adjacent to the HVC, as shown in Figure 2C. Birds in the second group also received infusion of carrier solution but were given testosterone-filled implants. Birds in the third group were given testosterone-filled implants but received an infusion of a neutralizing antibody to BDNF (α-BDNF). Hormonal treatments and infusion were carried out for 10 days, at which point the animals were sacrificed.

HVC volume was increased to a small but significant extent (18%) by testosterone treatment but was retained at its original level by α-BDNF infusion (P-value = 0.0155; Kruskal-Wallis test) (Figure 7). The HVC volume of the birds in this experiment that received only saline was larger than that of the controls shown in Figure 4. The two experiments were done on different years. We do not know what accounted for the differences in control HVC volume, but this measure is sensitive to histological processing, to time of year (in males), and possibly also to housing conditions. Importantly, each group of birds that underwent an experimental procedure had its own controls, kept under similar conditions and processed at the same time. The effects of testosterone that we report here are in the same direction as those reported in an earlier publication (Rasika et al., 1994).

The nuclear diameters of all HVC neurons were also increased by testosterone treatment, from 6.54 to 7.16 μm, but remained at 6.85 μm after α-BDNF infusion (P-value = 0.0019; Kruskal-Wallis test). The packing density of HVC neurons was decreased by 10 days of testosterone treatment, from 228,773 cells/mm^3 to 187,341 cells/mm^3, but remained high with α-BDNF infusion, at 235,729 cells/mm^3 (P-value = 0.0424; Kruskal-Wallis test). The total number of neurons was not significantly different between the three groups (P-value = 0.6126;

Figure 6. Effect of Testosterone Treatment on BDNF Levels in the HVC

Both the size of the HVC and immunostaining for BDNF are increased in testosterone-treated (T-treated) females when compared to controls, as revealed by perfusion with acetate-buffered saline (ABS [pH 3.0]) and paraformaldehyde.

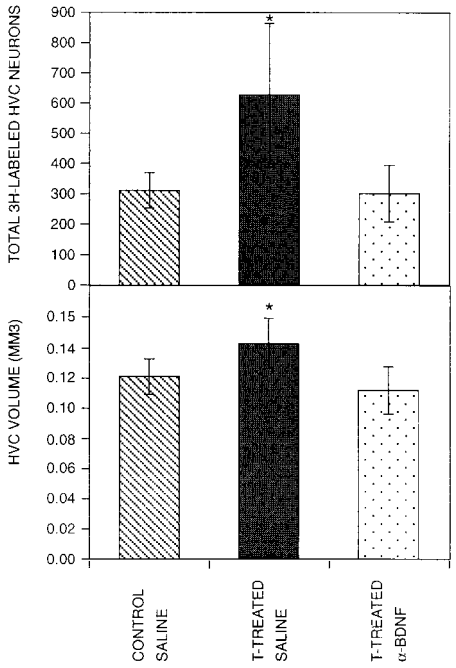

Figure 7. Effect of Hormonal Manipulation and Infusion of α-BDNF on the Total Number of New (^{3}H-labeled) Neurons in the HVC and HVC Volume

An asterisk (*) indicates a significant effect of treatment (ANOVA). Testosterone treatment alone results in the doubling of the total number of new or ^{3}H-labeled neurons and an 18% increase in the volume of the HVC, when compared to control females. However, α-BDNF infusion neutralizes this effect of testosterone treatment, retaining both the number of new neurons and HVC volume at control levels.

Kruskal-Wallis test), being 27,524 in the control group, 26,634 in the testosterone-saline group, and 26,369 in the testosterone-α-BDNF group.

The nuclear diameter of ^{3}H-thymidine-labeled neurons increased from 7.47 to 8.13 μm with testosterone treatment, but remained at 7.83 μm with α-BDNF infusion (P-value = 0.0077; Kruskal-Wallis test). The packing density of ^{3}H-thymidine-labeled neurons increased from 2,575 cells/mm^3 in controls to 4,298 cells/mm^3 in testosterone-treated females but was retained at 2,673 cells/mm^3 after α-BDNF infusion (P-value = 0.0090; Kruskal-Wallis test). There was no obvious gradient in the occurrence of labeled neurons with respect to distance from the infusion site. From the changes in volume and packing density, we infer that the total number of these new neurons in the HVC doubled after the 10 days of testosterone treatment. This testosterone-induced increase was prevented in the α-BDNF-infused females (P-value = 0.0087; Kruskal-Wallis test) (Figure 7).

Discussion

Our infusion experiment showed that the greater part of the neurons constantly added to the nucleus HVC of adult female canaries did not survive in the absence of exogenous BDNF. Systemic testosterone treatment led to an increase in the levels of BDNF in HVC. The effect of testosterone on survival of the new neurons was blocked by local infusion of α-BDNF. Taken together, these observations suggest that testosterone acts through BDNF to increase neuronal survival in HVC.

The Presence and Effect of BDNF in the HVC of Adult Canaries

There was an abundance of BDNF in the male HVC but little if any in females. The paucity of BDNF protein in the female HVC is striking in light of the fact that the full-length, active form of the BDNF receptor, TrkB, is present in both males and females. Minute amounts of BDNF in the female might be sufficient to act in a regulatory manner and warrant the expression of TrkB, or TrkB expression in females might be needed to respond to BDNF surges at certain times, e.g., during development or under certain adult conditions. However, TrkB is also the receptor for another ligand, NT-4/5 (Berkemeier et al., 1991), whose distribution in the adult canary brain is not known. NT-4/5, unlike BDNF, may be present in the HVC of both males and females, explaining TrkB expression in both sexes. However, our observations indicate that when BDNF is present in females, the new neurons recruited into HVC can respond to it, suggesting that BDNF is binding a functional receptor.

The dramatic effect of BDNF infusion on the recruitment of new HVC neurons could have resulted from new cells being rescued from death or attracted into HVC. Three lines of evidence suggest that the bulk of the effect is on cells that have ended their migration: (1) we saw no TrkB in cells—in HVC or elsewhere—with the morphology of migrating cells; (2) BDNF treatment started 25 days after the first ^{3}H-thymidine injection, which was meant to allow ample time for the majority of migrating cells to reach their destination (Alvarez-Buylla and Nottebohm, 1988; Al-Shamma and Arnold, 1997); and (3) there was no decrease in the density of new neurons in areas bordering the HVC, such as the shelf or cHV. This last observation suggests that the 3-fold, BDNF-driven increase in the number of new HVC neurons did not result from a rerouting of neurons that would have gone elsewhere but from the rescue of young neurons that, having reached their destination, in the absence of BDNF would have died. The magnitude of this rescue fits well with the observation that only a third of the neurons produced in the adult canary brain normally survive (Alvarez-Buylla and Nottebohm, 1988).

There was an 8% and 33% increase in the total number of HVC neurons of birds treated with BDNF in the spring and fall, respectively. These increases are congruent with our observation that BDNF treatment rescued cells that otherwise would have died. However, the observed increases were not significant, and larger sample sizes may be needed to determine whether or not BDNF leads to a net increase in HVC neuron number.

The Testosterone-BDNF Connection and Neuronal Survival

Both BDNF and testosterone (Rasika et al., 1994) increase HVC volume, nuclear diameters, and number of new neurons while reducing neuronal packing density.

This similarity is surprising given that, due to methodological constraints, the protocols used in the two studies differed. Two plausible interpretations come to mind: either the two molecules act through similar mechanisms, or BDNF mediates the effects of testosterone. The latter interpretation is supported by the fact that testosterone treatment increases BDNF immunopositivity in the HVC of females. If BDNF levels are secondary to those of testosterone, this would explain the sexual dimorphism in HVC's BDNF protein levels. Support for this view also comes from the observation that BDNF mRNA levels in the HVC of male canaries are higher in the spring than in the fall and apparently under the control of testosterone (S. R., F. N., and A. A.-B., unpublished data).

The effect of testosterone on BDNF expression could be direct or indirect. Examples of indirect effects could be their mediation by a testosterone metabolite, such as estradiol, or via a testosterone-induced increase in singing. Direct and indirect effects need not be mutually exclusive. Four different promoters have been found for the BDNF gene of mammals, allowing for multiple regulatory mechanisms (Timmusk et al., 1993; Nakayama et al., 1994). BDNF is highly conserved across species (Barde, 1994), and multiple regulation may occur in songbirds too. The mammalian BDNF gene is known to be regulated by estradiol (Singh et al., 1995; Sohrabji et al., 1995), but we do not know if this is also the case in canaries. It is known, however, that singing upregulates HVC's BDNF expression (Li et al., 1997). In the present study, all animals used for immunohistochemistry were blocked from singing for 2 hr before they were killed, yet the effect of singing on BDNF protein levels could have lasted longer than this.

The finding that BDNF infusion results in a dramatic increase in the survival of new neurons is not itself sufficient to say that BDNF is involved in neuronal rescue under normal circumstances. Such an effect could be merely a result of the presence of TrkB in HVC cells. Also, not all the new neurons recruited into the HVC need be responsive to testosterone or BDNF. Normal adult female canaries, which do not have appreciable levels of either substance, nevertheless show some neuronal recruitment, as indicated in this study and elsewhere (Goldman and Nottebohm, 1983; Rasika et al., 1994). Since there are at least two types of new neurons in the adult canary HVC, those that project to RA and others that might be local interneurons (Paton et al., 1985; Alvarez-Buylla et al., 1990a; Kirn et al., 1991), the possibility exists that different populations of new neurons depend upon different molecules for their survival. However, the result of blocking the testosterone-induced recruitment of new neurons by neutralizing the effects of endogenous BDNF suggests that BDNF is an important part of this process. Furthermore, in the relative absence of either endogenous testosterone or BDNF in female canaries, the finding that the effects of exogenous testosterone can be blocked by α-BDNF suggests that testosterone and BDNF act on the same population of new neurons and that testosterone acts by upregulating BDNF. BDNF might in turn enhance the effects of testosterone by increasing androgen receptors in the HVC, as has been reported for perineal motoneurons in mammals (Al-Shamma and Arnold, 1997).

Testosterone and BDNF given over a comparable period of time affect neuronal survival to different extents. Adult female canaries treated systemically with testosterone for 10 days achieve a mere 18% increase in HVC volume and a 2-fold increase in neuronal recruitment; BDNF infused next to HVC also for 10 days induces a 60% increase in HVC volume and a 3-fold increase in the number of labeled neurons. These two experiments cannot be readily compared because of the two different manners of drug administration. However, a possible inference is that systemic testosterone induces lower levels of BDNF in the HVC than direct infusion or that there is a delay between the rise in testosterone and the subsequent rise in BDNF levels. A separate study has shown that singing also upregulates BDNF in the HVC (Li et al., 1997). It is possible that under normal circumstances higher levels of BDNF are obtained in the HVC by the confluence of a direct effect of testosterone and an indirect, singing-mediated rise in BDNF. In our study, the birds in which we implanted osmotic pumps in the abdomen did not sing, despite the testosterone they received, yet there was in them a doubling in the number of new, ^{3}H-labeled neurons. Perhaps the latter increase would have been even greater had the birds sung. In either case, the increase was prevented by blocking BDNF.

Until now, the effects of neurotrophins on neuronal survival have been demonstrated primarily in vitro or in embryonic material. Changes in the level of a reproductive hormone regulate neuronal replacement in a part of the adult vertebrate brain that controls a learned behavior. Our observations suggest that this effect is mediated by BDNF.

Experimental Procedures

All animals used in this study were obtained from the close-bred colony of Waterslager canaries at the Rockefeller University Field Research Center. Animals were housed under New York State photoperiod. Food and water were available ad libitum. All protocols using live birds were approved by the Animal Care and Use Committee of the Rockefeller University, following National Institutes of Health guidelines for laboratory animal welfare.

Immunohistochemical Localization of BDNF and TrkB in Normal Male and Female Canaries

Four male and three female canaries, 1–2 years old in the spring (April), were anesthetized deeply by intramuscular injection of Nembutal (pentobarbital, 125 mg/kg body weight; Abbot Laboratories), and perfused transcardially with 0.9% saline followed by 3% paraformaldehyde in phosphate buffer (pH 7.4). Three male and three female canaries were anesthetized as described and perfused with Acetate-Buffered Saline (ABS; 5% acetic acid in 0.9% saline [pH 3.0]) (Zhou et al., 1994), followed by 3% paraformaldehyde in phosphate buffer (pH 7.4). Parasagittal sections 30 μm thick were cut from postfixed, cryoprotected brains on a freezing sliding microtome, washed in phosphate-buffered saline (PBS), and used on the same day.

Sections were blocked and incubated for 96 hr at 4°C in the primary antibody (sheep anti-mouse BDNF #AB1513P diluted 1:500; Chemicon) and for 2 hr at room temperature in the secondary antibody (donkey anti-sheep IgG diluted 1:50; Sigma). After incubation in ABC (ABC Elite Kit, Vector), the reaction was completed using 0.25 mg/ml diaminobenzidine (DAB) with nickel enhancement (Shu et al., 1988). Immunohistochemistry for TrkB was carried out as above, with the following changes: primary antibody consisted of rabbit anti-mouse TrkB (sc-12; Santa Cruz Biotechnology) diluted

1:500; secondary antibody consisted of goat anti-rabbit IgG (Vector Labs) diluted 1:200. Negative control sections in all cases were incubated with the diluent for the primary antibody, or nonspecific primary antibody. In addition, in the case of BDNF immunolabeling, primary antibody preabsorbed with a 50× excess of BDNF protein (Amgen) was also used to demonstrate specificity. No specific staining was seen in any of these cases.

Infusion of BDNF into the HVC

Four groups of three 1-year-old female canaries (two groups each for March and October) were injected intramuscularly with 50 μl of ^{3}H-thymidine (1 mCi/ml; New England Nuclear) twice daily for 5 days. 25 days after the first injection, osmotic pumps and cannulas were implanted into animals anesthetized by intramuscular injection of Nembutal (pentobarbital, 25 mg/kg body weight; Abbot Laboratories), followed by inhalation of Metofane (methoxyflurane; Mallinckrodt Veterinary). The flowrate of Alzet 1007D osmotic minipumps (Alza Corporation) was reduced to 0.4 μl/hr by immersing them in molten paraffin (Sigma) at 60°C to a depth of 7 mm (Vahlsing et al., 1989), and pumps were filled with 100 μl of 3 mg/ml human recombinant BDNF (Amgen) in a carrier of 0.9% saline with 20 μg/ml BSA (Sigma), or carrier alone. Such a setup delivers about 28.8 μg/day of BDNF, which is well within the range used by infusion studies in mammals (Mufson et al., 1994; Siuciak et al., 1996; Yurek et al., 1996). Cannulas were made from 30G (Becton Dickinson) needles and implanted lateral to the left HVC of each bird (Figure 2C). Pumps were implanted in the peritoneal cavity, with the delivery tube running outside the body, over the back and under the left wing of the animal. 10 days after infusion was started, animals were deeply anesthetized and perfused transcardially with 0.9% saline followed by 3% paraformaldehyde in phosphate buffer (pH 7.4).

Brains were embedded in polyethylene glycol (M_r 1500), cut at 6 μm intervals, processed for autoradiography, and stained with cresyl violet. The area of the HVC was calculated for every tenth section, and HVC volume estimated by multiplying the sum of these areas by the section thickness and sampling interval. Cells were mapped in four evenly spaced sections per HVC. All cells with large, lightly stained nuclei with one or two darkly stained nucleoli were counted as neurons (Goldman and Nottebohm, 1983). Neurons with at least 20 times the background level of exposed silver grains over their nucleus were identified and counted as ^{3}H-thymidine labeled (Figure 3). ^{3}H-thymidine-labeled neurons were also counted over four evenly spaced sections in the cHV (between the ventricle and LH), in the shelf (a region approximately 200 μm in width, below the HVC), and in the NCM. Whereas cHV and shelf were mapped in sections containing the HVC (i.e., at the same mediolateral position as the HVC), NCM was mapped in sections medial to the HVC, between 0.3 and 1.0 mm from the midline (Vates et al., 1996; Mello and Ribeiro, 1998). Nuclear diameters were obtained for 50 neurons per HVC, by measuring all neurons within a grid placed randomly over HVC sections. Nuclear size information was used to correct the frequency distribution of neurons in various size classes to compensate for the inclusion of smaller profiles that actually belong to larger neurons, and calculate the density of neurons according to size classes to compensate for larger nuclei being counted in multiple sections (Weibel, 1979; Cruz-Orive, 1983; Clark et al., 1990). Counts corrected in this manner were used to estimate total packing densities and neuron numbers. 25 labeled neurons per HVC (due to the paucity of labeled neurons compared to unlabeled ones, these were chosen by measuring all labeled neurons over HVC sections until the quota was reached), and all labeled neurons mapped in other regions, were also measured and their uncorrected nuclear diameter used to estimate the packing density and total number of labeled neurons (Clark et al., 1990).

Statistical comparisons between groups were carried out by factorial analysis of variance (ANOVA), with season and infusate as the two factors. Significance was independently confirmed by analyzing pairs of groups using the nonparametric Mann-Whitney U test.

Immunohistochemical Localization of BDNF in Control and Testosterone-Treated Female Canaries

Three 1-year-old female canaries were implanted subcutaneously in October with a Silastic implant containing testosterone, prepared by filling 5 mm of crystalline testosterone propionate into Silastic tubing (I. D. 0.76 mm, O. D. 1.65 mm; Dow Corning) and sealing the ends with Silastic glue (Dow Corning). Implants were incubated overnight in 0.9% saline in order to facilitate uniform release of testosterone upon implantation. Two control females received empty Silastic implants. Three weeks after implantation, animals were anesthetized deeply as described and perfused with ABS and 3% paraformaldehyde. Immunohistochemistry for BDNF was carried out as described above.

Infusion of α-BDNF into the HVC

Three groups each of five 1-year-old female canaries were injected twice daily for 5 days with ^{3}H-thymidine as described before. 25 days after the first injection, in October, osmotic pumps and cannulas were implanted into anesthetized animals; testosterone-filled or empty Silastic implants made as described above were subcutaneously implanted at the same time. The first group received osmotic pumps containing carrier (0.9% saline with 20 μg/ml BSA) and empty Silastic implants, the second group received osmotic pumps containing carrier, but testosterone-filled Silastic implants, while the third group received testosterone-filled Silastic implants and osmotic pumps containing 10 μg/ml of the BDNF antibody (AB1513P, Chemicon) in carrier. Animals were sacrificed 10 days after infusion was started, and tissue processing and quantitation of HVC volume, neuronal size, packing density, and number carried out as before. The nonparametric Kruskal-Wallis test was used for statistical comparisons between the three groups.

Acknowledgments

We thank Philippe Rousselot, Erich Jarvis, Sidarta Ribeiro, and Fiona Doetsch for technical advice, Pat Tellerday for organization and logistics, and Daun Jackson, Sharon Sepe, and Helen Ecklund for the care of animals as well as help with the injections. This work was supported by National Institutes of Health grants to F. N. and A. A.-B.

Received June 1, 1998; revised June 25, 1998.

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